Characterization of orally efficacious influenza drug with high resistance barrier in ferrets and human airway epithelia

Mart Toots, Georgia State University
Jeong-Joong Yoon, Georgia State University
Robert M. Cox, Georgia State University
Michael Hart, Georgia State University
Zachary M. Sticher, Emory University
Negar Makhsous, University of Washington
Roland Plesker, Paul Ehrlich Institute
Alec H. Barrena, Georgia State University
Prabhakar G. Reddy, Emory University
Deborah G. Mitchell, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: SCIENCE TRANSLATIONAL MEDICINE
Volume: Volume 11, Number 515
Publisher: AMER ASSOC ADVANCEMENT SCIENCE | 2019-10-23
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1126/scitranslmed.aax5866
Permanent URL: https://pid.emory.edu/ark:/25593/vqmdt

Final published version: http://dx.doi.org/10.1126/scitranslmed.aax5866

Accessed October 19, 2022 5:24 AM EDT
Characterization of orally efficacious influenza drug with high resistance barrier in ferrets and human airway epithelia


1Institute for Biomedical Sciences, Georgia State University, Atlanta, GA 30303
2Emory Institute for Drug Development, Emory University, Atlanta, GA 30322
3Virology Division, Department of Laboratory Medicine, University of Washington, Seattle, WA 98195
4Veterinary Medicine Division, Paul-Ehrlich-Institute, Federal Institute for Vaccines and Biomedicines, 63225 Langen, Germany
5Department of Pharmacology, Emory University, Atlanta, GA 30322

Abstract

Influenza viruses constitute a major health threat and economic burden globally, frequently exacerbated by pre-existing or rapidly emerging resistance to antiviral therapeutics. To address the unmet need of improved influenza therapy, we have created EIDD-2801, an isopropylester prodrug of the ribonucleoside analog N4-hydroxycytidine (NHC, EIDD-1931) that has shown broad anti-influenza virus activity in cultured cells and mice. Pharmacokinetic profiling demonstrated that EIDD-2801 was orally bioavailable in ferrets and non-human primates. Therapeutic oral dosing of influenza virus-infected ferrets reduced group pandemic 1 and group 2 seasonal influenza A shed virus load by multiple orders of magnitude and alleviated fever, airway epithelium histopathology, and inflammation, whereas post-exposure prophylactic dosing was sterilizing. Deep-sequencing highlighted lethal viral mutagenesis as the underlying mechanism of activity and revealed a prohibitive barrier to the development of viral resistance. Inhibitory concentrations were low-nanomolar against influenza A and B viruses in disease-relevant well-differentiated human air-

*Correspondence to: rplemper@gsu.edu.

Author contributions: Conceptualization: MT, GRP, RKP; data curation: MT, AAK, ALG, MGN, RKP; formal analysis: MT, RP, AAK, ALG, RKP; funding acquisition: GRP, RKP; chemical discovery: GRB, MGN, GRP; investigation: MT, JY, RMC, MH, ZMS, NM, AHB, PGR, DGM, RCS, ALG, RKP; project administration: MGN, RKP; resources: ALG, GRB, MGN, RKP; supervision: ALG, GRP, RKP; validation: AAK, ALG, MGN, RKP; visualization: MT, ALG, RKP; preparation of original draft: MT, RKP; review & editing: RP, AAK, ALG, MGN, GRP.

Competing interests: G.R.B., M.G.N. and G.R.P. hold patent 20190022116, “N4-Hydroxycytidine and Derivatives and Anti-Viral Uses Related Thereto”, covering composition of matter and method of use of EIDD-2801 for influenza therapy. This study could affect their personal financial status. All other authors declare no competing interests.

Data and materials availability: The distribution of influenza virus strains and non-commercial chemical material described in this study for research use is regulated by materials transfer agreements from Georgia State University and Emory University. All data from next-generation sequencing reads are deposited and accessible in the NCBI SRA under BioProject PRJNA528811. All other data is available in the main text or the supplementary materials.
liquid interface airway epithelia. Correlating antiviral efficacy and cytotoxicity thresholds with pharmacokinetic profiles in human airway epithelium models revealed a therapeutic window >1,713 and established dosing parameters required for efficacious human therapy. These data recommend EIDD-2801 as a clinical candidate with high potential for monotherapy of seasonal and pandemic influenza virus infections. Our results inform EIDD-2801 clinical trial design and drug exposure targets.

**One Sentence Summary:**

A next-generation broad-spectrum orally efficacious influenza A and B virus inhibitor is associated with a high genetic resistance barrier.

**Introduction**

Influenza viruses are segmented, negative polarity RNA viruses that spread through the respiratory route (1). The viruses are zoonotic and spillover especially of avian and swine origin viruses into the human population occurs frequently. The etiologic agent of the most recent influenza pandemic in 2009, for instance, was a triple reassortant influenza A virus (IAV) of swine origin (2,3). Even in interpandemic years, influenza viruses have major impact on human health and are responsible for over 600,000 death globally (4). In the 2017/2018 influenza season, seasonal influenza viruses caused close to 1 million hospitalizations, nearly 80,000 deaths, and loss of over 11 billion dollars in the United States alone (5), and created major clinical and economic burden globally. Morbidity and mortality during pandemics are substantially higher. The efficacy of current influenza vaccines is moderate under the best circumstances, but can fall below 20% (6). Vaccine efficacy is particularly low in the elderly, which are at greatest risk of developing severe, life-threatening disease.

Neuraminidase inhibitors are still standard of care (SOC) for influenza therapy, but are moderately efficacious and increasingly compromised by pre-existing resistance of circulating viruses (7). Resistance has entirely undermined the adamantes, which target the viral matrix-2 protein proton channel (8). The recently approved influenza virus polymerase acidic (PA) protein endonuclease blocker baloxavir marboxil was efficacious in clinical trials. However, resistant viruses emerged in 9.7% of trial participants within five days, sometimes coinciding with rebounding of symptoms and viral shedding (9). We recently demonstrated broad-spectrum activity of NHC against seasonal and highly pathogenic IAVs and influenza B viruses (IBVs) in mice and proposed viral error catastrophe as the basis for anti-influenza activity, although the exact molecular mechanism of compound action remained undefined (10). This study identifies a clinical candidate, EIDD-2801, establishes proof-of-concept for therapeutic efficacy far superior to that of SOC in two premier model systems of human influenza (ferrets and well-differentiated air-liquid interface (ALI) human airway epithelia), determines the mechanism of inhibition, and demonstrates a prohibitive genetic barrier against the emergence of resistant influenza viruses.
Results

An NHC prodrug orally available in non-human primates

Although NHC exhibited good oral bioavailability in rodents (10), plasma concentrations were low in cynomolgus macaques (Fig. 1A, table S1), which could reflect poor human bioavailability. In search of a broadly orally available therapeutic candidate, we synthesized EIDD-2801, the 5'-isopropylester of NHC (Fig. 1B), which showed increased oral bioavailability in non-human primates (Fig. 1A, table S1) and ferrets (table S2) compared to NHC. EIDD-2801 and NHC showed similar oral bioavailability in mice and EIDD-2801 was efficiently hydrolyzed in vivo after absorption, resulting in detection of only free NHC in plasma (fig. S1). Consequently, in vivo efficacy tests in this study used EIDD-2801, cell culture experiments were performed using NHC.

In vivo efficacy of EIDD-2801 in the ferret model of influenza infection

Ferrets recapitulate hallmarks of human influenza infection, providing a clinically-relevant animal model to investigate therapeutic intervention (11). To inform dose concentration selection, we determined single escalating dose oral pharmacokinetic (PK) profiles of EIDD-2801 in ferrets across a 4 to 512 mg/kg range. Total NHC plasma exposure (area under the curve from 0 to infinity (AUC\textsubscript{inf})) increased dose proportionally (linear correlation with R\textsuperscript{2} = 0.96) up to ~100 mg/kg dose (fig. S2, table S2). When EIDD-2801 was administered orally to ferrets at this highest concentration approximately associated with linear PK performance (128 mg/kg) and tissue distribution of NHC and its bioactive 5'-triphosphate anabolite NHC-TP were subsequently analyzed, we found sustained amounts of NHC-TP in respiratory tissues (>3.2 nmol/g lung tissue (table S3)), the major site of influenza virus infection, confirming efficient biodistribution and anabolism of NHC in ferrets. Overall exposure after single versus multi-dose oral administration of EIDD-2801 to ferrets, delivered in a twice-daily (b.i.d.) regimen, was dose-proportional, indicating that repeat dosing does not lead to drug accumulation (fig. S3, table S4). Seven b.i.d. doses at the 100 mg/kg concentration were well tolerated, causing no phenotypically appreciable adverse effects.

The highest dose associated with linear PK and 20% of that amount (100 and 20 mg/kg, respectively) were used in initial ferret efficacy tests. Animals were intranasally infected with 1 × 10\textsuperscript{5} plaque forming units (pfu) of 2009 pandemic strain A/California/07/2009 (H1N1) (Ca/09; (Fig. 1C–E)) or 1 × 10\textsuperscript{6} pfu of seasonal A/Wisconsin/67/2005 (H3N2) (Wi/05; (Fig. 1F–H)), representing IAVs of group 1 and 2 hemagglutinin subtypes, respectively. Treatment was initiated post-exposure prophylactically (12 hours post-infection; high dose only) or therapeutically (24 hours post-infection), after the onset of fever, the predominant clinical sign in Ca/09-infected animals, and continued b.i.d. to ensure trough NHC plasma concentrations ≥ 1 μM. Clinical signs were overall less prominent in Wi/05-infected animals. For all treatment groups, shed virus titers in nasal lavages were determined in 12-hour intervals, and fever monitored continuously using implanted telemetric sensors (Fig. S4). An additional two groups of Ca/09-infected animals were treated prophylactically (3 hours pre-infection), one receiving 100 mg/kg EIDD-2801, the other SOC oseltamivir-phosphate at approximately twice the human dose equivalent.
Infected control animals received vehicle (oral formulation as specified in Methods, but lacking EIDD-2801) at the identical gavage volumes. Independent of challenge strain, shed virus load in prophylactically or post-exposure prophylactically treated animals remained at or below limit of detection (Fig. 1C, F) and no signs of disease emerged (Fig. 1D, G). Dosed therapeutically at either concentration, EIDD-2801 reduced shed viral titers by ≥4 orders of magnitude within 24 hours of first dose, whereas prophylactically administered SOC oseltamivir had no significant (P ≥0.13) inhibitory effect on shed virus titer (Fig. 1C), consistent with previous observations (12, 13).

Duration of fever was significantly (P = 0.003) (Ca/09-infected group) shortened in therapeutically treated animals (Fig. 1D, G) and tissue viral titers were reduced by more than 4 orders of magnitude (Ca/09) or undetectable (Wi/05) 3.5 days after infection (Fig. 1E, H). Histological analyses of lung sections extracted at this time from Ca/09-infected animals revealed overall moderate pathological changes, similar to previous experience with the model (14). However, bronchiolar inflammation was more pronounced in oseltamivir and vehicle-treated ferrets than in the animal groups therapeutically treated with EIDD-2801 (Fig. 1I), resulting in significant differences (P = 0.015 and P = 0.025, respectively) in clinical scores (fig. S5).

**High genetic barrier to influenza virus escape from NHC**

Positive-strand RNA viruses encounter a high barrier to developing NHC resistance (15, 16). If equally applicable to influenza viruses, EIDD-2801 might offer a potential improvement for treating influenza therapy. We employed both dose-escalation and fixed-dose passaging strategies to induce IAV resistance to NHC. Gradual dose-escalation consistently resulted in virus extinction at drug concentrations ≥4 μM (fig. S6), equivalent to approximately 2-times the NHC EC$_{50}$ concentration against representative IAVs in MDCK cells (10). To test for evolution of population-wide resistance under continuous NHC pressure, we serially passaged virus populations in the presence of fixed drug concentrations. Whereas 4 and 10 μM NHC doses were rapidly sterilizing, 1 and 2 μM were tolerated for >10 passages (Fig. 2A and fig. S7). However, virus replication efficiency was impaired even at sublethal NHC concentrations (Fig. 2A). By comparison, signature resistance mutations to baloxavir marboxil at PA residue 38 (9) emerged readily and became rapidly allele-dominant in the population (Fig. 2B and Datafiles S1 and S2). Whole genome deep-sequencing of NHC- or vehicle-experienced virus populations after ten passages revealed only dose-dependent accumulation of random low frequency mutations (fig. 2B and Datafiles S3 and S4). Compared to the vehicle-treated populations, exclusively C-to-U and G-to-A transition mutations were significantly increased (P = 0.0003 and P < 0.0001) after ten passages in the presence of NHC (Fig. 2C), whereas relative frequencies of all other transition or transversion combinations were indistinguishable from the reference populations (fig. S8). Repeat exposure of all NHC-experienced virus populations to 4 μM NHC was again rapidly sterilizing (Fig. 2D), confirming that none of the random mutations mediates resistance to NHC. These results highlight lethal mutagenesis as the basis for influenza virus inhibition by NHC and reveal a high genetic barrier to resistance.
Antiviral activity in disease-relevant well-differentiated human airway epithelia cultures

To assess NHC efficacy in disease-relevant human tissues, we established a well-differentiated human airway epithelium model grown at ALI that displays hallmark airway epithelium features: differentiation into mucus-secreting goblet, ciliated, and basal cells, formation of tight and adherens junctions (Fig. 3A), and characteristic (17) increase in transepithelial/transendothelial electrical resistance (TEER) within three weeks (Fig. 3B). By eliminating dependence on exogenously added trypsin for proteolytic influenza virus hemagglutinin maturation, 3D-airway epithelia models are believed to best recreate physiological virus growth kinetics (11, 18, 19).

Whereas the infection of differentiated epithelia with IAV or IBV strains resulted in massive damage to the apical cell layer, treatment with 1.8 μM NHC in the basolateral chamber was sterilizing and preserved tissue integrity (Fig. 3C and fig. S9). LC-MS/MS analysis of epithelium extracts after exposure to 1.8 μM NHC revealed a corresponding NHC-TP concentration of approximately 255 pmoles/10^6 cells. Dose-response measurements against Ca/09, Wi/05, and B/Brisbane/60/2008 returned NHC EC_{50} values of 0.06-0.08 μM (Fig. 3D), whereas the EC_{50} concentration of SOC oseltamivir carboxylate was 0.19 μM (determined for Ca/09 only). These NHC EC_{50} values were ~25-50 fold lower in the human epithelia than in immortalized cell lines (10), which may reflect differences in viral infection kinetics and/or improved anabolism of NHC in differentiated ciliated cells, constituting the primary target for influenza virus in the epithelium (20). EC_{99} values, in our view representing a robust efficacy threshold, ranged from 0.15-0.25 μM basolateral NHC (Fig. 3D). Although IAV-induced tissue damage cannot be reversed in the epithelium model, therapeutic treatment with NHC initiated up to 72 hours after epithelium inoculation with Ca/09 significantly reduced (P = 0.009, P = 0.0002 and P < 0.0001) apically released virus load (fig. S10).

The 3D-airway epithelia tolerated continuous 3-day exposure to up to 50 μM basolateral NHC without TEER reduction (Fig. 3E), the predominant biomarker of tissue integrity (21), or microscopically appreciable changes in tight junction appearance (Fig. 3F). Regression modeling revealed a CC_{50} concentration of 137 μM NHC (fig. S11), which translates into a specificity index (SI = CC_{50}/EC_{50}) of ~1,713-2,283 against different influenza virus strains in human airway epithelium tissue, defining a large therapeutic window. These results establish approximately 0.2 μM and 50 μM basolateral NHC as robust efficacy and cytotoxicity thresholds in human airway epithelia. Anabolite analysis through mass spectrometry revealed that these prodrug concentrations correspond to extrapolated values of 28 and >1,500 pmoles NHC-TP/10^6 cells, respectively.

Treatment efficacy simulation based on physiological, dynamic inhibitor concentrations

Incorporation into host nuclear or mitochondrial RNA is a primary source of sub-phenotypic off-target effects of ribonucleoside analogs. We exposed primary HBTECs to 50 μM (cytotoxicity threshold), 10 μM, or 1.8 μM (sterilizing antiviral activity) NHC for 72 hours and assessed transition mutation frequency in nuclear and mitochondrial message. Whereas higher NHC concentrations increased C to U and U to C transitions in host mRNAs, no off-target effects emerged at the influenza virus-sterilizing dose of 1.8 μM NHC (Fig. 4A). Even

Sci Transl Med. Author manuscript; available in PMC 2020 October 23.
though the total number of transitions at the tested 1.8 to 50 μM NHC concentration range were not significantly different in nuclear versus mitochondrial mRNAs (P = 0.25 for 50 μM; P = 0.1 for 10 μM; and P = 0.2 for 1.8 μM), significantly greater (P = 0.0002) impact on mitochondrial than nuclear protein expression was observed (fig. S12). Differential sensitivity may reflect the proofreading inability of mitochondrial RNA polymerases compared to nuclear RNA Pol II (22). To examine sub-phenotypic effects in the context of efficacious therapy, we assessed mutation frequencies in ferret lung tissue after seven oral EIDD-2801 doses administered b.i.d. at 100 mg/kg. Transition frequencies in nuclear and mitochondrial message were indistinguishable between EIDD-2801 and vehicle-treated animals (Fig. 4B), consistent with the observed good tolerability of the drug.

To estimate dosing and treatment regimens expected to be required for efficacious human anti-influenza therapy, we recapitulated physiological NHC plasma PK profiles of three oral EIDD-2801 dose concentrations, 128 mg/kg, 20 mg/kg, and – extrapolated from the linear PK range shown in Fig. S2 – 7 mg/kg, in the basolateral chamber of the human airway epithelium cultures and determined NHC-TP tissue concentrations at 4 hours (anticipated \(C_{\text{max}}\)), 12 hours (trough in b.i.d. dosing), and 24 hours (for 128 mg/kg dose concentration only) after administration (Fig. 4C, fig. S13). Since repeat-dosing of ferrets had not resulted of NHC build-up in plasma, drug concentration oscillations between doses were described by consecutive plotting of measured peak and trough NHC-TP concentrations (Fig. 4D). Overlaid with the robust anti-influenza efficacy and cytotoxicity thresholds, this simulation predicted that consistent dosing with 7 mg/kg oral EIDD-2801 b.i.d. will yield sustained tissue NHC-TP concentrations in close range of the 250 pmoles/10^6 cells-threshold of sterilizing anti-influenza virus activity without sub-phenotypic off-target effects.

We tested this prediction in ferrets, first by treating Ca/09-infected animals therapeutically with 7 mg/kg EIDD-2801 b.i.d. after a single 20 mg/kg loading dose. To assess signature upper respiratory pro-inflammatory cytokine and chemokine expression status associated with influenza virus infection of ferrets, nasal turbinates were extracted from these animals 2.5 days after infection, corresponding to near peak response-time point in the upper respiratory tract (23, 24). Shed virus load in EIDD-2801-treated animals dropped by several orders of magnitude within 24 hours (Fig. 4E) and fever started to decline in treated animals approximately 1.5 days after infection (Fig. 4F). However, termination of this study 2.5 days after infection prevented informative statistical analysis of the effect of treatment on fever duration. Relative cytokine (P = 0.003) and CXCL10 induction (P = 0.009) was significantly lowered (Fig. 4G). CXCL10 in particular has been implicated in pathophysiological effects resulting from pulmonary virus infection (25).

**Effect of therapeutic treatment on viral spread and lung disease**

These efficacy results closely mimicked the effect size of higher EIDD-2801 dose concentrations. We therefore omitted the loading dose and assessed upper and lower respiratory tract viral burden 3.5 days after infection of animals with Ca/09 and therapeutic administration of EIDD-2801 at the 7 mg/kg b.i.d. concentration. Shed virus load (Fig. 5A) and duration of fever (Fig. 5B) were significantly (P <0.0001 and P = 0.03, respectively) reduced, confirming the predictions of the ex vivo tissue model. Consistent with potent...
antiviral activity, cellular infiltrates in the nasal cavities (Fig. 5C) and blood lymphocyte counts (Fig. 5D) remained unchanged in treated animals over the duration of the study. Significantly lower shed virus load (P < 0.0001) in EIDD-2801-treated animals was corroborated by a reduction of several orders of magnitude in virus burden in nasal turbinates and tracheas of animals in the treatment group (Fig. 5E).

Assessing viral spread to the small airways, we noted that therapeutic administration of EIDD-2801 significantly reduced viral titers in bronchoalveolar lavages (P = 0.02) and lung tissue (P = 0.009) sections (Fig. 5E). To account for positional effects of virus replication in the lung (26), virus titers were assessed in four lung lobes – left and right caudal and cranial – for each animal (Fig. 5F). Immunohistochemical assessment of nasal turbinates from two EIDD-2801 and two vehicle-treated animals showed extensive infection of the epithelium layer in the vehicle group that was largely absent in the treated animals (Fig. 5G and fig. S14). Viral infection of the lung was near immunohistochemical detection limit in the vehicle group, which is consistent with low lung virus signal intensities reported in previous studies of pandemic (H1N1) 2009 viruses in the model, in which equivalent lung titers had been reached (27, 28). Virus was immunohistochemically undetectable in lung sections from EIDD-2801-treated ferrets (Fig. 5H and fig. S15), underscoring the effective suppression of virus lung invasion.

Discussion

We have identified a ribonucleoside analog, EIDD-2801, with good oral bioavailability across multiple species and broad-spectrum anti-influenza efficacy. The inhibitor combines potent reduction of clinical signs and virus titers shed from the upper respiratory tract with reduced virus spread to the small airways when administered in a therapeutic dosing regimen. EIDD-2801 compared favorably to current SOC oseltamivir phosphate, which in our study and previously reported work (12, 13) exhibited only very moderate efficacy against pandemic 09 H1N1 virus even when administered prophylactically. The alleviated lung histopathology observed in the EIDD-2801-treatment group furthermore underscored that efficacious dose concentrations do not cause appreciable tissue damage in vivo.

Our next-generation sequencing analysis of EIDD-2801-experienced virus populations indicated a high genetic barrier to influenza virus resistance to inhibition in cell culture. This observation was reminiscent of slowly emerging influenza virus escape from T-705 (favipiravir) (29), a nucleoside analog currently approved in Japan for use against pandemic influenza viruses that do not respond to conventional therapies. However, a potential for teratogenicity (30) compromises the clinical use of favipiravir especially against seasonal influenza, and we noted that anabolism of favipiravir to the bioactive tri-phosphate is inefficient in some primary human cells of disease-relevant respiratory origin (10). Although it was originally thought that escape of influenza virus from favipiravir was unlikely (31, 32), a recent study combining continued virus passaging in the presence of sub-sterilizing compound concentrations with deep sequencing revealed a 2-step escape pathway (33). Over the course of ten passages, a virus population emerged in which a mutation in the polymerase basic 1 (PB1) subunit mediated resistance to favipiravir, albeit at a fitness cost, which was compensated for by a second mutation in the PA subunit. The equivalent
adaptation approach applied in our study to EIDD-2801 did not yield any polymerase allele variations that became fixed in the virus population, providing promise that EIDD-2801 addresses a major limitation of current influenza virus therapeutics, although only prolonged viral exposure to the compound in vivo will ultimately reveal whether some degree of resistance can be achieved.

Deep-sequencing of an EIDD-2801-experienced virus population furthermore revealed that the compound caused specifically C-to-U and G-to-A transition mutations in influenza virus genomes, demonstrating that tautomeric interconversions of NHC (34) base pair as cytosine or uracil at replication or transcription once NHC has been integrated into the viral RNA. Notably, A-to-G transition mutations were absent in genomes of NHC-experienced influenza viruses, although we had previously observed that transcripts of respiratory syncytial virus, a member of the distantly related pneumovirus family of non-segmented RNA viruses (35), harbored C-to-U, G-to-A, and A-to-G transitions after exposure to NHC (10). We consider it most likely that initial recognition of NHC-TP by orthomyxovirus and pneumovirus polymerases for incorporation into nascent RNAs is distinct. Based on transition mutation characteristics, influenza virus polymerase appears to accept NHC at the stage of incorporation only in place of cytosine and not uracil, and tautomeric interconversion must therefore occur subsequently at the replication stage. In contrast, respiratory syncytial virus polymerase appears capable of incorporating NHC in either tautomeric form, resulting in transitions originating from primary incorporation of NHC-TP in place of uracil and subsequent base pairing as cytosine (A-to-G), post-incorporation tautomeric interconversion and base pairing as uracil (G-to-A), and tautomeric interconversion of NHC incorporated in progeny viral genomes prior to reverse transcription and sequence analysis (C-to-U).

These results are consistent with the induction of viral error catastrophe (36) as the predominant mechanistic principle of NHC activity influenza viruses and other RNA viruses such as respiratory syncytial virus. However, we cannot rule out at present that the compound may have additional, non-mutagenic effects that inhibit virus replication. Favipiravir, for instance, was found to be incorporated by influenza virus polymerase both as a guanosine and an adenosine analog, consistent with the induction of error catastrophe, but tandem incorporation of two favipiravir moieties reportedly prevents further transcript extension in an in vitro influenza virus polymerase assay (37). We demonstrated that substitution of cytidine triphosphate for NHC-TP partially restores respiratory syncytial virus polymerase activity in a comparable in vitro assay (10), but it remains to be addressed whether NHC increases the frequency of delayed chain termination or interrupts RNA extension after tandem incorporation by influenza virus polymerase.

The ferret model recapitulates major features of human influenza (11), but naturally cannot replace an evaluation of drug anabolism, stability, and antiviral performance in human tissues. To narrow the recognized gap between efficacy assessment in animals and clinical testing (38–40), we correlated animal model-derived insight into physiologically achievable dynamic prodrug concentrations with drug performance in well differentiated human air-liquid interface airway epithelia cultures. This approach supported the identification of efficacy and safety thresholds in a disease-relevant human tissue model, revealing in treatment simulations a viable window for efficacious human influenza therapy. Efficacious
dose concentrations extracted from the model were experimentally validated in the ferret model, underscoring the predictive power of the system.

In conclusion, our data identify EIDD-2801 as a promising clinical candidate for monotherapy of seasonal and pandemic influenza. Although SI values defined by the human airway epithelia model are encouraging and no gross signs of toxicity were observed in the corresponding ferret efficacy studies, formal two-species multi-day toxicity and adverse event testing will determine first-in-human dose concentrations. With an understanding of the drug safety profile in humans gained in Phase 1 clinical trials, treatment simulation models developed in this study will aid in establishing dosing paradigms for future clinical efficacy testing.

Materials and Methods

Study design

The overarching objective of the study was to explore preclinical efficacy and pharmacokinetics of ribonucleoside analog EIDD-2801 against influenza viruses in the ferret animal model and disease-relevant human 3D airway epithelium models, and establish key concentration target thresholds for future clinical evaluation. The efficacy models were chosen because they recapitulate hallmarks of human influenza and therefore constitute premier systems to evaluate the efficacy of drug candidates. The effect of treatment on virus replication and clinical signs in ferrets was examined at different oral dose concentrations using prophylactic and therapeutic treatment regimens. Treatment was considered efficacious when statistically significant reduction in virus titers in nasal lavages and respiratory tissues was observed, and the duration of clinical signs was shortened. Efficacy and cytotoxicity of the drug candidate in the 3D human airway epithelium models was assessed by four-parameter variable-slope regression modeling of 50% inhibitory and cytotoxic concentrations, respectively, and TEER measurements. Timeline endpoints were predefined before initiation of experiments. At least three animals per condition were used in all pharmacokinetics and in vivo efficacy experiments. Prior to initiation of each individual study, animals were randomly assigned to treatment and control groups. Over the course of this project, one animal reached IACUC-defined humane endpoints prematurely due to a complication unrelated to infection or treatment and was removed from analysis. Exact numbers of biological repeats for all experiments are specified in figure legends. No blinding was used. At least three biological replicates were examined per group and experiment, specific numbers of biological and technical replicates are included in the figure legends. Primary numerical data is shown in Datafile S5.

Cell lines and transfections

Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS). Normal primary human bronchial tracheal epithelial cells (HBTECs) (purchased from LifeLine Cell Technology) from a 30-year old healthy female donor were grown in BronchiaLife cell culture medium (LifeLine Cell Technology). These cells were obtained by the vendor under informed consent and adheres to the Declaration of Helsinki,
The Human Tissue Act (UK), CFR Title 21, and HIPAA regulations. All regulatory approval lies with the vendor. Immortalized cell lines used in this study were routinely checked for microbial contamination (in approximately 6-month intervals). HBTECs were tested for microbial contamination on July 25, 2017 by LifeLine Cell Technology. Only HBTECs with a passage number 1-4 were used for this study.

**Influenza viruses**

A/WSN/33 (H1N1) expressing nanoLuc reporter gene (described in (41)), A/California/7/2009 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Brisbane/60/2008 were propagated on MDCK cells for 2-3 days at 37°C. Viruses were titrated by TCID$_{50}$-hemagglutination (TCID$_{50}$-HA) assay on MDCK cells as described in (10). Virus stocks were generated by collecting progeny virions from cell culture supernatants cleared by centrifugation (4,000 rpm for 20 min at 4°C), and stored in aliquots at −80°C.

**PK in cynomolgus macaques**

Non-naïve cynomolgus macaques (4 males, 4 females; 2 to 6 years of age) retrieved from the colony stock at Concord Biosciences and originally received from Charles River Laboratories were each dosed orally with 100 mg/kg of NHC dissolved in 240 mM citrate buffer, followed by blood collection from the femoral vein at the specified time points. After a 7-day washout period, each animal was dosed orally with 130 mg/kg of EIDD-2801 dissolved in 80% (v/v) PEG-400, 20% (v/v) N,N-Dimethylacetamide, followed by blood collection from the femoral vein at the specified time points. In a separate study, non-naïve cynomolgus macaques (3 males, 3 females; 2 to 6 years of age) retrieved from the colony stock at MPI, Inc and originally received from Charles River Laboratories were each dosed intravenously with 10 mg/kg of NHC dissolved in 0.9% sterile sodium chloride, followed by blood collection from the femoral vein at the specified time points. For all samples, plasma was separated from heparinized blood and stored at −80°C before analysis as described in NHC (10). For calibration, standard curves were prepared in blank plasma (concentrations range 10 to 10,000 ng/ml). Quality-control samples of 30, 500, and 5000 ng/ml in blank plasma were analyzed at the beginning of each sample set. Calibration showed linearity with R$^2$ values >0.99.

**PK and PD in ferrets**

Female ferrets (6 to 8 months of age) received from Marshall BioResources were rested for one week, assigned randomly to study groups and dosed orally with EIDD-2801 dissolved in 1% methylcellulose, followed by blood collection from the anterior vena cava and tissue sampling at the specified time points. Three animals per groups were sampled for PK analyses and 2-3 animals for PD testing. Plasma was separated from heparinized blood, and tissue samples snap-frozen and stored at −80°C before analysis as described in NHC (10). For calibration, standard curves were prepared in blank plasma (concentrations range 10 to 100,000 ng/ml) and blank tissue lysate (concentration range 1.56 to 3,130 ng/ml), respectively. Quality-control samples of 30, 500 and 5,000 ng/ml in blank plasma were analyzed at the beginning of each sample set. Calibration in each matrix showed linearity with R$^2$ values >0.99.
Influenza infection studies in ferrets

Female ferrets (6 to 8 months of age) were received from Marshall BioResources and housed in an ABSL-2 (animal biosafety level) facility. Ferrets were rested for 1 week, weighed, assigned randomly to groups, anesthetized with dexmedetomidine/ketamine, and infected intranasally with $1 \times 10^5$ (A/California/07/2009 (H1N1)) or $1 \times 10^6$ pfu (A/Wisconsin/67/2005 (H3N2)); infection volume 200 μl. Treatment with EIDD-2801 was initiated 3 hours before infection (prophylactic regimen), 12 hours post-infection (post-exposure prophylactic regimen), or 24 hours post-infection (therapeutic regimen), and continued for 3.5 days b.i.d. Compound was administered orally in 3.5 ml doses in 1% methylcellulose formulation and chased with 3.5 ml high-calorie liquid dietary supplement. Control groups received vehicle (1% methylcellulose in water) volume equivalents. Body temperature was monitored continuously (readings every 2-15 minutes) using implanted telemetric sensors (e-Celsius Medical monitoring system from BodyCap Medical). Bodyweight of animals was measured at start and end of each experiment, and for some experiments daily; no changes in body weight were detected. Additional monitoring of phenotypically appreciable adverse effects included assessment of animals for changes in overall composure, activity level or vocation and occurrence of diarrhea, vomiting or reduced food uptake. Viral load was measured from nasal lavages (collected in 12-hour intervals) and nasal turbinates (upper respiratory tract), harvested 3.5 days after infection if not specified otherwise.

Histopathology

Lungs were perfused using 10% neutral-buffered formalin (10% formalin, 4g/L NaH$_2$PO$_4$ 6.5g/L Na$_2$HPO$_4$), dissected, and fixed for 24 hours. Formalin-fixed lungs were embedded in paraffin, sectioned (5 μm thickness), and stained with hematoxylin and eosin. Slides were evaluated by a licensed pathologist. Pathology score range 0 - 3 was used to evaluate tissue damage: 0 – no obvious changes in the lung; 1 – slight increase of cell count in the lung interstitium, mild bronchiolar inflammation; 2 – presence of granulocytes in the bronchial lumen of wall, moderate bronchiolar inflammation; 3 – substantial increase of cell count and widening of the interstitium, severe bronchiolar inflammation.

Virus adaptation

MDCK cells were infected with A/WSN/33-nanoLuc or Ca/09 at MOI of 0.001. Escalating dose adaptations used increasing concentrations (0.25 – 4 μM) of NHC, doubled at each passage; equivalent adaptations of Ca/09 to baloxavir marboxil were carried out in parallel. For fixed concentration adaptations, A/WSN/33-nanoLuc was passaged up to 10-times in the presence of 1, 2, 4 or 10 μM NHC or volume-equivalent DMSO. NanoLuc readouts (ranging from $10^7$-$10^3$) were measured after every passage to determine the presence of virus. Luciferase activity readouts were used to normalize the virus inoculum before every passage across the individual passage series; normalization was capped at a maximum of 15% of supernatant volume applied to re-infection. In all cases, viral RNA was extracted after each passage; specified passages were subjected to next-generation sequencing. Virus titers were evaluated through TCID$_{50}$-HA assay as described (10). Virus was considered eliminated when no infectious virus titer was detectable, nanoLuc readouts were below 5x10$^3$, and no
PB1-specific PCR product was detected after RT-PCR. For adaptations to baloxavir marboxil, infected MDCK cells received a loading dose of 0.9 to 100 nM of baloxavir marboxil, followed by escalating dose adaptation with concentrations specified in Fig. 2B.

**Next-generation sequencing**

Extracted viral RNA were treated with Turbo DNase I (Thermo Fisher) according to the manufacturer’s instructions. First strand cDNA synthesis used 19 μl of DNased RNA primed with 1 μl of 50 μM oligo(dT) and SuperScript III reverse transcriptase (Thermo Fisher), according to the manufacturer’s instructions. Single-stranded cDNA was cleaned up using Zymo DNA Clean and Concentrator (Zymo Research) and eluted into 10 μl of ddH₂O. Double-stranded cDNA was generated using cDNA from the previous step using 1 μl of 150 ng/μl random hexamers and Sequenase 2.0 (Thermo Fisher). Double-stranded cDNA was purified using Zymo DNA Clean and Concentrator and eluted in 10 μl of ddH₂O. NGS libraries were prepped with 2 μl of the double stranded cDNA using two-fifth Nextera XT volumes with 22 cycles of PCR amplification. Amplified libraries were purified using 0.8 Ampure XP beads. Libraries were run on a 1.2% agarose FlashGel (Lonza) for quality control and quantified using Qubit 2.0 (Thermo Fisher). Libraries were run on 1×192bp and 2×300bp runs on an Illumina MiSeq. Sequencing reads are available in NCBI BioProject PRJNA528811.

Because oligo-dT sequencing does not recover high amounts of coverage at the 5′end of the genes, coverage at the PA I38T/I38M locus in the baloxavir marboxil-experienced samples was relatively low. We therefore used a specific RT-PCR-Nextera approach to deep sequence this locus in baloxavir marboxil- and DMSO-passaged virus. RT-PCR of the 5′end of the PA gene was performed on extracted viral RNA using the Qiagen One-Step RT-PCR kit with the specific primers PA-F (CAA AAT GGA AGA CTT TGT GCG AC) and PA-R (GGA ATC CCA TAG ACT CCT AC), using 35 cycles of PCR with a 55°C Tm and 30 second extension time. The PCR products were purified and subjected to dual-indexed library preparation using the Nextera XT kit (Illumina). Only read 1 of a 2×300bp Illumina MiSeq run was used to determine allele frequencies. Read 1 FASTQ files were quality and adapter-trimmed using cutadapt (available at https://doi.org/10.14806/ej.17.1.200) and aligned to the DMSO-passaged reference IAV genome with bwa-mem using default options and Picard PCR deduplication. Allele frequencies at I38 were called using bcftools call --vm options on the mpileup using the ADF, ADR, and DP4 fields. PCR-Nextera sequencing reads are available in NCBI BioProject PRJNA528811.

**Analysis of next-generation sequencing data for antiviral resistance selection**

Raw fastq reads were first quality filtered and adapter trimmed using cutadapt. To interrogate samples passaged in NHC for potential resistance alleles, a custom pipeline, Longitudinal Analysis of Viral Alleles (LAVA), was used. LAVA is available at https://github.com/michellejlin/lava. Briefly, LAVA takes multiple longitudinal samples as fastq files, aligns them to the passage 1 consensus using bwa (42) removing PCR duplicates with Picard then calls all variants in coding regions with VarScan (43) and subsequently converts these changes into amino acid changes with Annovar (44). LAVA outputs HTML visualizations of minor allele changes over time across all samples. Since IAV has multiple
segments, the passage 1 consensus genome was created by concatenating the 8 IAV H1N1 reference segments from assembly GCA_000865725.1 with 100 Ns as a spacer between each segment. The passage 1 DMSO fastq file for each sample was mapped to this reference. Next, the majority consensus of this alignment was called and the original fastq reads were realigned to the new consensus to determine allelic changes relative to the passage 1 sample. LAVA output html file of alleles with AF >5% for DMSO, NHC 1 μM, and NHC 2 μM passage 10 samples for four biological replicates compared to the sample 1 DMSO passage 1 genome is provided in supplementary Dataset file. Variants were not called in the C-terminal luciferase portion of the polymerase basic 2 (PB2) segment.

Analysis of nucleotide changes in viral RNA

To further interrogate the NHC mechanism of action, the frequency of all nucleotide changes in viral RNA was analyzed. In an attempt to minimize the impact of sequencer error, additional quality control steps were performed on these reads: firstly, raw fastq reads were adapter and quality trimmed using cutadapt as above, single end reads were quality filtered removing any read that had any base with a quality score below 34, and read files were run through the LAVA pipeline to obtain VCF files for each sample; secondly, custom python scripts were used to parse the VCF files. In order to exclusively sample non-directed changes any mutational change occurring at greater than 5% allele frequency was discarded. The frequency of every possible nucleotide change was calculated for each sample and adjusted to represent the rate of change per 10,000 bases. Transition rates were compared to an average of the DMSO control for each treatment and passage number. The VCF parsing and analysis code is available at https://github.com/rcs333/frequency-scanner.

Establishment of 3D human airway ALI models

3.3 × 10^4 HBTECs at a passage number ≤3 were seeded onto 6.5 mm Costar Transwell Cell Culture Inserts (pore size 0.4 μm) and grown submerged in BronchiaLife cell culture medium to 100% confluency. Upon confluency, media was removed from the apical chamber and the media in the basal chamber changed to HBTEC Air-Liquid Interface Differentiation Medium (LifeLine Cell Technology). The cells were grown at air-liquid interface (ALI) for at least 21 days to ensure complete differentiation; media in the basal chamber was changed every 2 days. The developing 3D cultures were washed apically weekly to remove excess mucus. Transepithelial/transendothelial resistance (TEER) was measured using an EVOM volt/ohm meter coupled with an STX2 electrode (World Precision Instruments). Cultures with resistance ≥700 Ω/cm² were used for experimentation.

Confocal microscopy

3D human airway epithelial cells were fixed with 4% paraformaldehyde-PBS for 20 minutes followed by permeabilization with 0.5 % TritonX-100-PBS for 2 hours. Nonspecific protein binding sites were blocked 5% BSA-PBS for 1 hour. After blocking, the cells were incubated with primary antibody in antibody-binding solution (2% BSA-PBS) for 1 hour, washed 3 times with PBS, followed by incubation with conjugate antibody for 45 minutes. All antibodies are specified in Table S5, and all steps were performed at room temperature in the dark. Membranes were placed on glass slides using a mounting medium supplemented with 0.1 mM 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes), covered with a
coverslip, and edges sealed with nail polish. A Zeiss LSM 800 confocal microscope coupled with AiryScan module was used for detection, Zeiss Zen Blue software was employed for image analyses.

**Influenza infection and NHC treatment in airway 3D ALI model**

3D human airway epithelial cells were apically infected with influenza A or B viruses (5,000 pfu/transwell) for 2 hours, followed by washing of the apical chamber 3-times with media. Treatment with NHC or vehicle volume-equivalents was initiated from the basal chamber, mimicking NHC concentrations in plasma after oral dosing. Shed virus was collected from the apical chamber every 24 hours. Treatment efficacy was evaluated by TCID\textsubscript{50}-HA titration of shed virus and four-parameter variable slope regression modelling. Three wells were used for each concentration and time measurement.

**Assessment of transitions in host mRNA**

For detection of transition mutations in host cell mRNA, HBTECs were exposed to the specified NHC concentrations for 3 days; volume-equivalent of DMSO was used as vehicle control. For detection of transitions after in vivo exposure, ferret lung lobes from animals dosed orally in a b.i.d. regimen at 100 mg/kg NHC for 3.5 days were harvested, total RNA extracted using the ZR viral RNA kit (Zymo Research), and cDNA synthesized with SuperScript III reverse transcriptase (Thermo Scientific) and oligo(dT) primers. Nuclear SDH-A (HBTECs) or TNF\textalpha{} (ferrets), and mitochondrial COX-1 (HBTECs) or COX-15 (ferrets) sequences were amplified from the cDNAs and subcloned using CloneJet PCR Cloning Kit (Thermo Scientific). A minimum of 7 subclones (corresponding to a total of at least 5,000 nucleotides) from each sample were Sanger sequenced. Transition frequencies were expressed per 5,000 nucleotides and statistical significance was evaluated using Fisher’s exact test. Primer sequences are specified in Table S6.

**Assessment of host nuclear and mitochondrial gene expression—HBTECs**

were exposed to NHC at the specified concentrations for 3 days; volume-equivalent amounts of DMSO served as vehicle control. MitoBiogenesis In-Cell ELISA (Abcam; ab110217) was used to evaluate host mitochondrial (COX1 gene) and nuclear (SDH-A) gene expression according to the manufacturer’s instructions. Five to 17 subclones (corresponding to a total of at least 5,000 nucleotides) from each sample were Sanger sequenced. Signals were normalized to vehicle-treated samples and CC\textsubscript{50} values calculated through four-parameter variable-slope regression modeling. Primer sequences are specified in supplementary Table S6.

**PK and PD recapitulation in 3D human airway models**

3D human airway cultures were exposed basolaterally to 1.8 \mu{}M (sterilizing antiviral efficacy) NHC for 24 hours. To determine human tissue NHC-TP concentrations corresponding to in vivo PK analyses, 3D cultures were exposed basolaterally to the dynamic NHC concentrations detected in ferret plasma samples after 128, 20, or – extrapolated – 7 mg/kg oral dosing (concentrations specified in Fig 4C and fig. S13. Cells were lysed with 70% methanol at the specified timepoints, supernatants cleared by centrifugation at 13,000×g for 10 minutes at 4°C, and samples kept at −20°C until analysis.
NHC and its anabolites were analyzed by a qualified LC/MS/MS method as described in NHC (10). Three transwells were used for each measurement.

**Cytokine expression profile in ferret lungs**
Relative pro-inflammatory cytokine induction in ferret lungs were determined by real-time PCR analyses. Female ferrets were infected with Ca/09 and treated therapeutically with EIDD-2801. RNA was extracted from lung tissues 2.5 days post-infection; uninfected animals were used as controls. cDNA reverse transcribed with SuperScript III was analyzed by real-time PCR using Fast SYBR green master mix (Applied Biosystems). Signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, analyzed by the comparative threshold cycle (ΔΔCt) method, and expressed relative to uninfected animals (equivalent to day 0 of infection). Sequences of the primers used for the analyses are shown in Table S6.

**Immunohistochemistry**
Nasal turbinates and lung tissues were fixed with 10% neutral-buffered formalin for 24 hours, followed by decalcification for 5 days (nasal turbinates only), embedding into paraffin blocks, and sectioning (5 μm thickness). Slides were deparaffinized and antigens recovered using HistoReveal (Abcam). Endogenous biotin was blocked by incubating the slides in 0.001% biotin-PBS for 10 minutes, followed by 10-minute incubation in 0.001% avidin-PBS. Slides were then blocked with BSA Blocker (Thermo Scientific), incubated with specific anti-IAV HA antiserum (Table S5) overnight at 4°C, washed twice with PBS, and incubated with biotinylated conjugate antibody. Signal was detected using the ABC Peroxidase Staining Kit (Thermo Scientific) according to the manufacturer’s instructions and DAB staining with hematoxylin counterstaining.

**Ethics statement.**
All animal work was performed in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the Animal Welfare Act Code of Federal Regulations. Experiments involving ferrets and mice was approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) under protocols A18035 and A17019, respectively. Cynomolgus macaques studies were approved under USAMRMC protocol CB-2014-53.04, IACUC protocol number 036070.

**Statistical analyses**
One-way ANOVA (when more than two groups were compared) with Dunnett’s or Sidak’s multiple comparison post hoc tests, unpaired t-test (when only two groups were compared), or 2-way ANOVA with Dunnett’s or Sidak’s multiple comparison post hoc tests were used to assess statistical difference between samples. Statistical differences in the duration to resolve of fever were explored through time-to-event Log-rank (Mantel-Cox) tests. Fisher’s exact test was used for analysis of transition events in host message. All statistical analyses were carried out in GraphPad Prism software. Specific tests applied to individual data sets are specified in the corresponding figure legends. Individual biological replicates for all graphical representations are shown in the figures and the number of biological repeats is
specified in the figure legends. Representation of means or medians ± SDs of experimental uncertainty are shown, and specified in figure legends. Organ distribution for 12-hour time points in table S3 was based on two ferrets and shows average ± range. Valid two-sided testing was applied with a significance threshold (α) set to 0.05. Exact P values are provided in the figures. No adjustments were made to alpha levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank V von Messling for lending the telemetric ferret body temperature measurement system and sharing ferret qPCR primer sequences, V Edupuganti, L Moellerig and JA Marlow for assistance with pharmacological analyses, MT Saindaine and MA Lockwood for chemical syntheses, K Ganti and AC Lowen for counselling on immunohistochemistry procedures, and AL Hammond for critical reading of the manuscript.

Funding: This work was supported, in part, by contracts HDTRA1-15-C-0075 (to GRP) and HHSN272201500008C (to GRP) from the DTRA and the NIH/NIAID, respectively, by Public Health Service grants AI071002 (to RKP), AI119196 (to RKP), and HD079327 (to RKP) from the NIH/NIAID and NIH/NICHD, respectively, and by a pilot grant from the Georgia Research Alliance (to GRP and RKP). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

References:


40. van Meer PJK, Kooijman M, Gispen-de Wied CC, Moors EHM, Schellekens H, The ability of animal studies to detect serious post marketing adverse events is limited. Regul Toxicol Pharm 64, 345–349 (2012).
Fig. 1. Anti-influenza efficacy of EIDD-2801 in ferrets.

(A) Plasma PK profiles of NHC and therapeutic candidate EIDD-2801 in cynomolgus macaques (N = 8) after 100 mg/kg oral dose. F indicates oral bioavailability. (B) 2D structures of EIDD-2801 and its hydrolysis product, NHC. (C-I) Ferrets infected intranasally with Ca/09 (C-E, I; N = 3-6) or Wi/05 (F-H; N = 3-7) were treated orally with 100 or 20 mg/kg EIDD-2801 b.i.d. for 3.5 days post-infection. Shed (C, F) and nasal turbinate (E, H) virus titers are shown. (D, G) Body temperature was continuously monitored telemetrically. Lowes analysis of data obtained from all animals/group. Prophylactic treatment of Ca/09-
infected animals with oseltamivir is shown for comparison. Symbols in (A-H) represent biological repeats, graphs indicate medians ± SD; 2-way ANOVA (shed titers) or 1-way ANOVA (nasal turbinate titers) with Dunnett’s post hoc test. Differences in resolve of fever were assessed through time-to-event Mantel-Cox test. LoD – limit of detection; MTFR – median time to fever resolve. (I) Representative images of lung sections.
Fig. 2. Resistance profiling.
(A) Virus titers after passages shown in fig. S7 (N = 3). (B) Adaptation of influenza virus to baloxavir marboxil and NHC. Adaptation profile and amino acid frequencies of resistance mutations after deep-sequencing of baloxavir marboxil-experienced (N = 2) viruses (top panel). Deep-sequencing to identify mutation frequency (cut-off 5%) in influenza virus polymerase components after ten passages at 1 or 2 μM NHC or vehicle (lower panels). (C) C-to-U and G-to-A transition events after five and ten passages at 1 or 2 μM NHC relative to vehicle. Symbols show biological repeats, columns are means and error bars represent SDs. Statistical significance was explored by 2-way ANOVA and Dunnett’s post hoc test. (D) Repassaging of NHC-experienced virus populations from (A) at 4 μM NHC. Symbols represent
biological repeats, lines connect means. N = 4 for all NHC and vehicle-experienced virus populations in B-D.
Fig. 3. Anti-IAV and IBV efficacy in well-differentiated human airway epithelium cultures.

(A) Confocal microscopy 21 days post-ALI induction, showing hallmarks of airway epithelia: tight junctions (anti-ZO-1), adherens junctions (anti-E-Cadherin), goblet cells (anti-Muc5AC), and ciliated cells (anti-β-tubulin). Nuclei stained with DAPI. (B) TEER measurements throughout the 21-day differentiation at ALI. Symbols represent individual transwells (N = 10), line shows mean; 2-way ANOVA with Dunnett’s post hoc test. (C) Ca/09 or B/Brisbane/60/2008-infected airway epithelia cultures, treated basolaterally with 1.8 μM NHC or DMSO volume equivalents. Stained are viral antigens (Ca/09: anti-NS1; B/ Brisbane/60/2008: anti-IBV), tight junctions, and nuclei. (D) NHC dose-response curves against IAV and IBV in 3D epithelia cultures. Oseltamivir tested against Ca/09 only (N = 3-6/concentration point). Apically shed virus was harvested three days post-infection, EC<sub>50</sub> calculations through four-parameter variable-slope regression modeling. Symbols show biological repeats, lines connect means. (E) TEER after 3-day exposure to 50 μM NHC or vehicle (N = 3). Line connects means. (F) Confocal microscopy of samples from (E), showing tight junctions and nuclei. Enlarged immunofluorescence images in fig. S9 and fig. S16–17.
Fig. 4. Simulation of influenza therapy in well-differentiated human airway epithelium models. (A) Transitions in HBTEC nuclear (SDH-A) and mitochondrial (COX1) mRNAs after 3-day NHC treatment; ≥5 clones and ≥5,000 nucleotides/concentration examined; Fisher’s exact test was applied. (B) Transitions in ferret lung nuclear (TNFα) and mitochondrial (COX15) mRNAs after 7 oral b.i.d. EIDD-2801 doses at 100 mg/kg; 11 clones and ≥7,000 nucleotides were examined. (C) Recapitulation of 128 mg/kg EIDD-2801 oral ferret NHC plasma PK profile in the basolateral chamber of well-differentiated human airway epithelium cultures. NHC concentrations applied is shown in gray columns, corresponding NHC-TP concentrations were measured by LC-MS/MS after 4, 12, and 24 hours. Symbols show biological repeats (N = 3/time point). (D) NHC-TP concentrations in human airway epithelia recapitulating oral EIDD-2801 b.i.d. treatment at 128, 20 or 7 mg/kg in ferrets. Solid lines connect measured NHC-TP from (C) and fig. S13, dashed lines extrapolate b.i.d. treatment, dotted lines mark robust efficacy (EC₉₉), sterilizing antiviral activity, and cytotoxicity thresholds. (E-G) Ca/09-infected ferrets treated therapeutically with 7 mg/kg oral EIDD-2801 b.i.d. after a single 20 mg/kg loading dose (LD) (E) Shed viral load. Symbols (N = 3) show biological repeats, lines connect medians; 2-way ANOVA with Sidak’s post hoc test. (F) Lowes analysis of continuously monitored body temperature. Differences in resolve of fever were assessed through time-to-event Mantel-Cox test. MTFR – median time to fever resolve. (G) Select cytokine and chemokine mRNA induction in nasal turbinates 2.5
days post-infection; values are relative to uninfected animals, symbols show biological repeats (N = 3), columns means ± SD; Welch’s unequal variances t-test was applied.
Fig. 5. Experimental validation of simulation results.
Ca/09-infected ferrets treated therapeutically with 7 mg/kg EIDD-2801 b.i.d for 3.5 days.
(A) Shed viral load. Biological repeats (N = 5) are shown, lines connect medians; 2-way ANOVA with Sidak’s post hoc test. (B) Lowes analysis of body temperature. Differences in resolve of fever were assessed through time-to-event Mantel-Cox test. MTFR – median time to fever resolve. (C) Total number of cells in nasal lavages. (D) Total white blood cell counts. Symbols in (C) and (D) show biological repeats (N = 5), lines connect means; 2-way ANOVA with Sidak’s post hoc test. (E) Virus titers in upper and lower respiratory tracts 3.5 days after infection. Symbols show biological repeats (N = 3); graphs indicate medians ± SD; individual statistical assessments of titers in the distinct respiratory tract compartments with unpaired t-tests. NT: nasal turbinates; BALF: bronchioalveolar lavage fluid. (F) Virus

Sci Transl Med. Author manuscript; available in PMC 2020 October 23.
distribution in individual lung lobes. Symbols show titers in caudal and cranial lung lobes for individual animals; graphs indicate medians ± SD. (G) Immunohistochemistry of nasal turbinates from vehicle and EIDD-2801-treated animals. Specific detection with anti-IAV HA antiserum and DAB staining, hematoxylin counterstain. Red arrows mark isolated DAB-positive cells detected in treated animals. Scale bars represent 100 μm (overview microphotographs) and 25 μm (inserts), representative fields of view are shown. (H) Immunohistochemistry of lung sections from vehicle and EIDD-2801-treated animals. Staining and size bars as in (G).